Exhibit 9

Defective regulation of the epithelial Na^+ channel by Nedd4 in Liddle's syndrome

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Liddle's syndrome is an inherited form of hypertension linked to mutations in the epithelial Na^+ channel (ENaC). ENaC is composed of three subunits (α, β, γ), each containing a COOH-terminal PY motif (xPPxY). Mutations causing Liddle's syndrome alter or delete the PY motifs of β- or γ-ENaC. We recently demonstrated that the ubiquitin–protein ligase Nedd4 binds these PY motifs and that ENaC is regulated by ubiquitination. Here, we investigate, using the *Xenopus* oocyte system, whether Nedd4 affects ENaC function. Overexpression of wild-type Nedd4, together with ENaC, inhibited channel activity, whereas a catalytically inactive Nedd4 stimulated it, likely by acting as a competitive antagonist to endogenous Nedd4. These effects were dependant on the PY motifs, because no Nedd4-mediated changes in channel activity were observed in ENaC lacking them. The effect of Nedd4 on ENaC missing only one PY motif (of β-ENaC), as originally described in patients with Liddle's syndrome, was intermediate. Changes were due entirely to alterations in ENaC numbers at the plasma membrane, as determined by surface binding and immunofluorescence. Our results demonstrate that Nedd4 is a negative regulator of ENaC and suggest that the loss of Nedd4 binding sites in ENaC observed in Liddle's syndrome may explain the increase in channel number at the cell surface, increased Na^+ reabsorption by the distal nephron, and hence the hypertension.

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Introduction

The amiloride-sensitive epithelial Na^+ channel (ENaC) plays an essential role in the reabsorption of fluid across epithelia of the kidney, colon, lung, and ducts of exocrine glands. It facilitates the entry of Na^+ across apical membranes, a process driven by the basolateral Na^+/K^+-ATPase (1). Hence, it has a major impact on the control of whole body Na^+ homeostasis, blood volume, and blood pressure. The activity of the Na^+ channel is tightly controlled by various hormones such as aldosterone, vasopressin, and insulin, a number of intracellular mediators such as Na^+, Ca^{2+}, pH, cAMP, enzymes such as protein kinases A and C, and by extracellular proteases (1). Precise regulation of ENaC is essential, as illustrated by the number of human diseases that have been linked to malfunction or to mutations in ENaC, including Liddle's syndrome (2), pseudohypoaldosteronism type I (PHA-I) (3), cystic fibrosis (4), and pulmonary edema (5, 6). Liddle's syndrome is an autosomal dominant form of inherited human arterial hypertension, characterized by an early onset of severe hypertension, salt sensitivity, hypokalemia, metabolic alkalosis, and low aldosterone and renin plasma concentrations (7, 8). The disease has been genetically linked to mutations in either of two genes encoding the β or γ subunits of the ENaC complex (2, 9).

ENaC is composed of a combination of three similar subunits, α, β, and γ (10–13), assembled with a stoichiometry of 2α, 1β, 1γ (14). Each subunit consists of short intracellular NH_2 and COOH termini, two transmembrane domains, and a large extracellular loop (15–17). The COOH-terminal region of every subunit contains conserved proline-rich sequences, including the sequence xPPxY, called the PY motif (18). The original mutations identified to cause Liddle's syndrome, including frameshifts and premature stop codons, all lead to truncation of the COOH terminus, with deletion of the PY motifs of β- or γ-ENaC (2, 9). Moreover, in some kindred, specific point mutations within the PY motif were recently reported (19–21), pointing to this region as an important site for the regulation of ENaC and as a Liddle's syndrome "hot spot." Indeed, an abnormal increase in channel activity was observed upon expression of ENaC harboring the Liddle's truncation or mutations within the PY motif of β- or γ-ENaC in *Xenopus* oocytes (22–24). This elevated ENaC activity has been shown to be caused by both an increase in the number of channels at the plasma membrane as well as an elevated mean open probability of each channel (25), and it was recently linked also to a defective Na^+-dependent feedback inhibition (26).

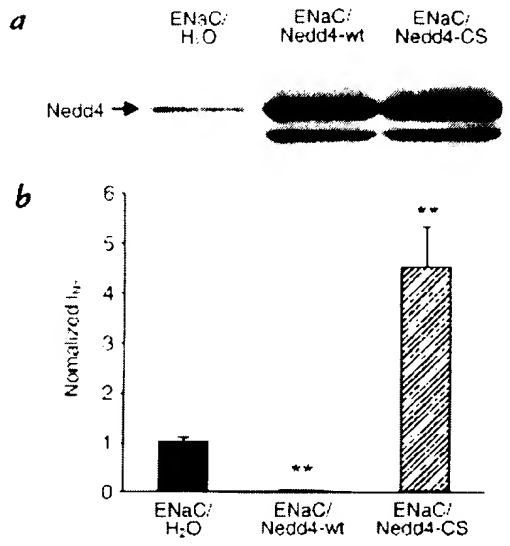

Figure 1

Regulation of ENaC by Nedd4 overexpressed in *Xenopus* oocytes. (***a***) Immunoblot analysis showing expression of xNedd4 in oocytes injected with cRNA of ENaC alone (*ENaC/H₂O*), or ENaC coinjected with either 25 ng of xNedd4-wt cRNA (*ENaC/Nedd4-wt*) or the same amount of mutant xNedd4 lacking ubiquitin-protein ligase activity (*ENaC/Nedd4-CS*). Proteins from oocyte lysates were separated on SDS-PAGE and immunoblotted with anti-xNedd4 antibodies. The left lane shows the level of endogenous xNedd4. Faster-migrating protein bands in Nedd4-injected oocytes likely represent degradation products. (***b***) I_{Na} were measured in oocytes expressing either ENaC alone (*ENaC/H₂O*), ENaC plus xNedd4-wt (*ENaC/Nedd4-wt*), or ENaC plus xNedd4C938S (*ENaC/Nedd4-CS*). The measured currents were normalized to control oocytes (4.2 ± 0.6 μA/oocyte). $n = 36$ oocytes from six different batches. $^{**}P < 0.01$ vs. ENaC/H₂O-injected oocytes. *ENaC*, epithelial sodium channel; I_{Na}, amiloride-sensitive Na^+ current; *wt*, wild-type.

We had previously identified the ubiquitin protein ligase Nedd4 (neuronal precursor cell expressed developmentally downregulated) as an interacting partner of ENaC, which binds to the PY motifs of αβγENaC via its WW domains (27). This protein, originally cloned by a subtractive screen (28), contains a C2/CalB domain (a calcium-dependent lipid-binding domain), three WW domains (29) (but four in the human and *Xenopus laevis* homologues) that are protein-protein interaction modules, and a HECT domain (homologous to E6-AP protein COOH-terminal, a ubiquitin-protein ligase domain) (30). Because ubiquitination of proteins has been associated with their rapid breakdown by the proteasome or by the endosomes/lysosomes in the case of some transmembrane proteins (31), we proposed that Nedd4 may act as a negative regulator of ENaC, which upon binding via its WW domains to the ENaC PY motifs, ubiquitinates the channel, leading to its subsequent endocytosis and lysosomal degradation (27). Indeed, we found that ENaC is a short-lived protein that is ubiquitinated *in vivo* and regulated by ubiquitination (32). Mutation of key lysine residues at the NH_2 termini of γ- and α-ENaC led to reduced ubiquitination and elevated channel activity when expressed in *Xenopus* oocytes, an elevation caused by an increase in the number of channels at the plasma membrane (32). Furthermore, we have demonstrated that the pattern of expression of Nedd4 in kidney and lung (33) is similar to that previously reported for ENaC (34).

Despite these lines of evidence, however, it has not yet been demonstrated directly that Nedd4 actually regulates ENaC function, which was therfore the goal of our current studies. Here we report that Nedd4 indeed negatively regulates ENaC activity and that this regulation is dependent on the presence of the PY motifs of ENaC and on Nedd4 with functional ubiquitin-protein ligase activity. Moreover, we find that Nedd4 affects ENaC activity predominantly by controlling the number of channels at the plasma membrane. This study provides an explanation at the molecular level for at least one aspect of the defective regulation of ENaC in Liddle's syndrome.

Methods

Plasmids and constructs. Rat ENaC constructs were cloned into the pSD5 plasmid (10). All the ENaC constructs have been described previously by Schild *et al.* (24). A full-length *Xenopus laevis* Nedd4 (xNedd4) (35) clone was produced by generating an *Xba*I site (silent mutation, by replacing the codon CGG encoding R297 with the codon AGA) and joining a 5′ and a 3′ cDNA clone together. A catalytically inactive xNedd4 cDNA clone was generated by mutating cysteine 938 to serine (xN4C938S) by PCR. Wild-type (wt) and mutant xNedd4 were then cloned into a pSDeasy plasmid, a variant of pSD5. The GenBank/EMBL/DDBJ accession number for the xNedd4 sequence is AJ000085, and the number for rat Nedd4 is U50842.

Expression and function of ENaC channels in Xenopus oocytes. Rat ENaC and *Xenopus* Nedd4 constructs were transcribed using SP6-RNA polymerase, and 10 ng cRNA encoding ENaC (3.3 ng of each subunit) with or without 25 ng cRNA (or as indicated) encoding xNedd4 were coinjected into oocytes. Amiloride-sensitive Na^+ currents (I_{Na}) were measured by the two-electrode voltage-clamp method as described previously (32). Binding experiments were performed according to Firsov *et al.* (25), with both β- and γ-ENaC containing a FLAG epitope tag in their ectodomains. All values were normalized to the mean values in one given batch of oocytes. Data are presented as mean ± SEM. The statistical significance of the differences between the means was estimated using bilateral Student's *t* test for unpaired data.

Biochemical analyses. After the electrophysiological measurements, oocytes were kept at 4°C, pooled, and lysed in (20 μl/oocyte) Triton X-100 homogenization buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin) at 4°C. After centrifugation at 4°C for 10 min, the supernatant was recovered and stored at −80°C. For Western blot analysis, 40 μl of lysate was separated on 8% SDS-PAGE, transferred onto nitrocellulose, and then immunoblotted as described before (27), using an anti-xNedd4 antibody at a dilution of 1:1,000. The anti-xNedd4 antibodies were raised in rabbits against a COOH-terminal 72-kDa protein fragment that was expressed and purified as a histidine-tagged protein.

Immunofluorescence with anti-FLAG antibody. Twenty-four hours after injection of the α-ENaC subunit with both β- and γ-ENaC containing a FLAG epitope, oocytes were fixed with 3% paraformaldehyde in PBS for 4 h. Cryosections (6 μm) of fixed oocytes were obtained and performed as described by Mastroberardino *et al.* (36). For immunocytochemistry, a tyramide signal amplification (TSA-Direct) kit (Du Pont NEN Research Products, Boston, Massachusetts, USA) was used according to the manufacturer's instructions. ENaC FLAG was detected

with an anti-FLAG IgG antibody (Eastman Kodak, Rochester, New York, USA) that was diluted 1:100 in the TSA Blocking buffer. Sections were rinsed with PBS containing 0.05% Tween (PBS-Tween) and were subsequently incubated with a 1:100 dilution of horseradish peroxidase conjugated sheep anti mouse Ig (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA). After repeated washing, binding sites of the secondary antibody were revealed with FITC-tyramide conjugates diluted 1:50 in TSA amplification diluent. Sections were washed with PBS-Tween and were mounted in DAKO-glycergel (DAKO, Glostrup, Denmark). All antibody incubations were done for 1 h at room temperature. Sections were studied by epifluorescence and images were acquired with a VISICAM CCD camera (Visitron, Puchheim, Germany).

Results

ENaC is regulated by Nedd4 in Xenopus oocytes. We have shown previously that the ubiquitin–protein ligase Nedd4 binds ENaC (27) and that ENaC is a short-lived protein regulated by ubiquitination (32). This prompted us to investigate whether Nedd4 is indeed a regulator of ENaC activity. For these studies, we used *Xenopus laevis* oocytes, which contain endogenous Nedd4 (ref. 27; see also Fig. 1*a*, *left lane*). We thus injected rat ENaC cRNA in *Xenopus* oocytes together with cRNA encoding wild-type *Xenopus laevis* Nedd4 (xNedd4) (35) or a mutant form bearing a Cys→Ser mutation (xN4C938S) at the conserved cysteine in the HECT domain, which inactivates its ubiquitin–protein ligase activity (30, 37). Both wild-type and mutant xNedd4 were overexpressed to similar levels, about 10- to 20-fold higher than that of endogenous xNedd4 (Fig. 1*a*). The overexpression of xNedd4 did not influence the biosynthesis of ENaC, as judged by metabolic labeling of *Xenopus* oocytes followed by immunoprecipitation with anti-ENaC antibodies (not shown). ENaC activity was determined by measuring amiloride-sensitive Na^+ currents (I_{Na}) 20–24 hours after cRNA injection and oocyte incubation in a solution containing 80 mM sodium. Expression of ENaC alone (ENaC/H_2O) in *Xenopus* oocytes (control) resulted in I_{Na} between 1 and 10 μA. Upon coexpression of xNedd4-wt with ENaC (ENaC/Nedd4-wt), I_{Na} fell below 5% of the control values (Fig. 1*b*); this channel inhibition was dose-dependent, becoming more pronounced with increasing amounts of xNedd4 cRNA injected (Fig. 2). In contrast, coexpression of the inactive Nedd4 mutant (ENaC/Nedd4-CS) resulted in a two- to sixfold fold increase in I_{Na} relative to control oocytes. Expression of this mutant Nedd4 did not lead to obvious changes in other electrophysiological parameters, such as amiloride-sensitive current–voltage relationship, single-channel properties of ENaC, and non-amiloride-sensitive membrane conductance (not shown). Moreover, the activity of another channel of the ENaC/degenerin superfamily, FMRFamide peptide-gated Na^+ channel (FaNaCh; ref. 38), which has no PY motifs, was not influenced by the expression of either wild-type or mutant xNedd4. In the same experimental conditions as for ENaC, the FaNaCh associated I_N were 5.0 ± 1.8 μA/oocyte in control oocytes (FaNaCh cRNA injected alone); I_{Na} reached 4.3 ± 1.0 and 5.2 ± 1.2 μA/oocyte with expression of the Nedd4-wt and mutant Nedd4, respectively (n = 16 for each groups, in two batches of oocytes). Taken together, these results demonstrate that Nedd4 inhibits ENaC activity.

The Nedd4-mediated downregulation of ENaC is dependent on the presence of the PY motifs. We have shown previously that the interaction between Nedd4 and ENaC occurs via Nedd4-WW domains binding to the PY motifs in ENaC (27). Therefore, we tested whether the functional regulation of ENaC by Nedd4 is also dependent on the presence of intact PY motifs. To this end, we expressed a mutant ENaC channel in which the tyrosines of the PY motifs of all three subunits were mutated to alanines (in the case of α- and γ-ENaC), or to histidine in β-ENaC (Fig. 3); this ENaC mutant (ENaC-ΔPY_3) was thus missing all the binding sites for Nedd4 (27). When expressed in oocytes, such a channel showed an approximately fourfold increase in activity, as compared with ENaC-wt channels (Fig. 3, compare black columns). This elevated channel activity was unaltered by coexpression of either Nedd4-wt or of the inactive xN4C938S Nedd4 (Fig. 3), demonstrating that the PY motif–mutated ENaC has lost its responsiveness to Nedd4. Because deletion/mutation of the PY motifs of either β- or γ-ENaC is sufficient to cause Liddle's syndrome, we tested the responsiveness of such a Liddle's channel to Nedd4. Thus, we coexpressed Nedd4-wt or the xN4C938S mutant together with ENaC bearing a COOH-terminally truncated β-subunit (βR564stop), an originally described Liddle's mutation (2). Our results show that this Liddle's channel was only partially responsive to Nedd4-wt, with an effect intermediate between ENaC-wt possessing all PY motifs and ENaC lacking all of them (Fig. 3). Quantitatively, this βR564stop channel showed an approximately threefold increase in activity when compared with

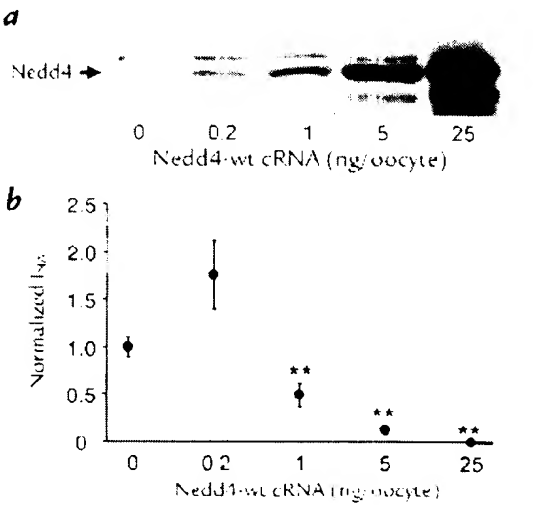

Figure 2
Dose response relationship of the effect of Nedd4 on ENaC activity. (*a*) Immunoblot analysis showing expression of xNedd4 with increasing amounts of xNedd4 cRNA (ng/oocyte). (*b*) Corresponding normalized I_{Na}. The measured currents were normalized to control oocytes (6.1 ± 2.3 μA/oocyte). n = 16–18 oocytes from three different batches. $^{**}P < 0.01$ vs. ENaC/H_2O injected oocytes (*i.e.*, 0 ng of xNedd4 cRNA). The difference in I_{Na} in oocytes injected with 0 and 0.2 ng cRNA/oocyte was not statistically significant.

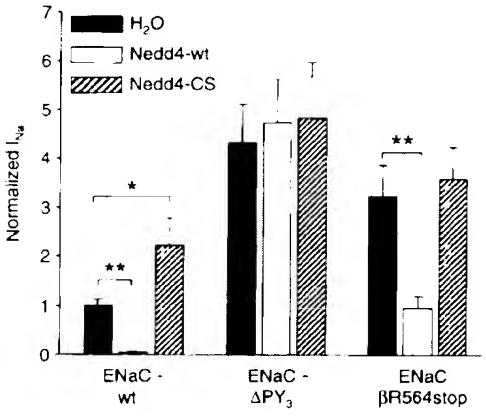

Figure 3
ENaC PY motifs are necessary for the effect of Nedd4 on ENaC activity. Oocytes were either injected with ENaC-wt (*left three bars*), ENaC bearing mutations in all three PY motifs (ENaC-ΔPY_3; *middle three bars*), or a Liddle's ENaC channel missing the COOH terminus of β-ENaC (*βR564stop*), including its PY motif (*right three bars*), together with either H_2O (*closed bars*), Nedd4-wt (*open bars*), or xNedd4C938S (*hatched bars*). Currents were normalized to control mean values (5.5 ± 1.2 μA/oocyte). Twenty-four oocytes from four different batches were measured per condition. $^*P < 0.05$ and $^{**}P < 0.01$ represent levels of significance relative to conditions indicated by the brackets.

ENaC-wt (Fig. 3), and its coexpression with Nedd4-wt yielded only 71.5 ± 2.0% inhibition of I_{Na} compared with 95.1 ± 0.3% inhibition of ENaC-wt (this difference was significant, with $P < 0.001$; $n = 24$). Moreover, coexpression of the xN4C938S Nedd4 mutant with the βR564stop channel did not lead to the stimulation of channel activity seen with ENaC-wt. Thus, these data demonstrate that the absence of even one PY motif, as seen in Liddle's syndrome, is sufficient to interfere with Nedd4 regulation of ENaC, and that the greater the number of PY motifs (*i.e.*, Nedd4-WW binding sites) present within the channel, the tighter the control of Nedd4 on ENaC activity.

Nedd4 affects the number of channels at the plasma membrane. Because we demonstrated regulation of ENaC activity by Nedd4, we next investigated the mechanism(s) by which this regulation is accomplished. There are three possible mechanisms by which Nedd4 could affect ENaC activity: (*a*) the number of channels at the plasma membrane, (*b*) the open probability of the channel, and (*c*) the single-channel conductance. To quantitate the number of channels at the cell surface, we expressed ENaC channels that were FLAG- tagged at the extracellular loops of the β and γ subunits ($ENaC^f$) (25). This allowed quantification of binding of ^{125}I-labeled anti-FLAG antibodies (*i.e.*, determination of the number of channels at the cell surface) and simultaneous measurement of I_{Na} in the same oocyte, as described by Firsov *et al.* (25). As seen in Fig. 4, the anti-FLAG antibody binding was affected by Nedd4 proportionally to the macroscopic changes in I_{Na}, suggesting that it is primarily the number of channels at the cell surface that is controlled by Nedd4. This is different from the βR564stop (Liddle's) channel, where Firsov *et al.* had previously demonstrated that the increase in ENaC numbers could only account for about half of the total increase in channel activity, and changes in open probability were also contributing to this elevated activity (25). To further substantiate these findings, we performed immunostaining of the injected oocytes using anti-FLAG antibodies that stain $ENaC^f$. As can be seen in Fig. 5, in control oocytes expressing ENaC alone, ENaC-related immunofluorescence was visible in the plasma membrane as well as in a mainly reticular, intracellular compartment. When ENaC was coexpressed with xNedd4-wt, ENaC-related immunofluorescence was no longer detectable at the plasma membrane, and the staining intensity in the intracellular compartments seemed to be reduced, suggesting that ENaC was not only internalized, but also more rapidly degraded. In contrast, when the inactive Nedd4 mutant (xNC938S) was coexpressed with ENaC, ENaC-related immunostaining was drastically increased at the plasma membrane, whereas staining intensity in the intracellular, reticular compartment appeared to be unaffected, suggesting retention of ENaC at the cell surface and impairment of channel internalization and degradation. In control experiments with uninjected oocytes, no staining was observed (not shown; see also ref. 36). Collectively, these studies show that Nedd4 regulates the number of ENaC channels at the plasma membrane.

Discussion

The goal of the present study was to provide a direct demonstration of the regulation of ENaC by Nedd4 and the implications of this regulation for Liddle's syndrome. The *Xenopus laevis* oocyte represents a convenient experimental system to study such questions, as we and others have previously shown that elevated Na^+ channel (ENaC) activity can be observed when channels containing Liddle's syndrome mutations are expressed in these oocytes

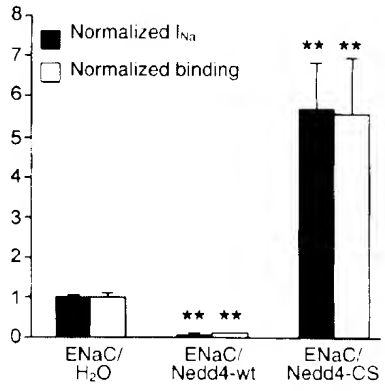

Figure 4
Nedd4 regulates the number of ENaC channels at the cell surface. Oocytes were coinjected with $ENaC^f$ and either H_2O (*left two bars*), 25 ng xNedd4-wt cRNA (*middle two bars*), or xNedd4C938S (Nedd4-CS; *right two bars*). To quantitate the number of channels at the cell surface, I_{Na} (*closed bars*) and binding of iodinated anti-FLAG antibodies (*open bars*) were measured in the same oocytes, as described previously (25). Current and binding values were normalized to control values (5.5 ± 1.2 μA oocyte and 0.10 ± 0.01 fmol/oocyte, respectively). $^{**}P < 0.01$ vs. $ENaC^f$, H_2O injected oocytes.

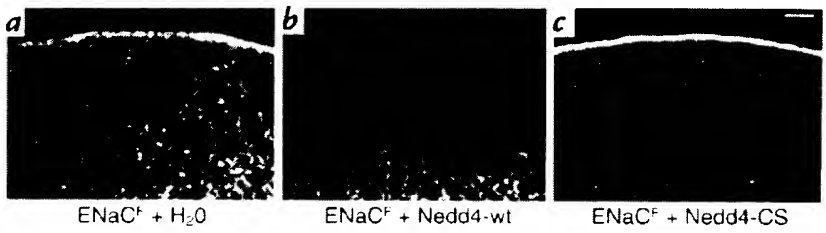

Figure 5
Immunostaining of ENaC expressed in *Xenopus* oocytes coexpressing Nedd4-wt or -CS. ENaC^F-related membrane immunostainings were carried out with anti-FLAG antibodies in oocytes expressing ENaC alone (***a***), ENaC plus Nedd4-wt (***b***), or ENaC plus xNedd4C938S (Nedd4-CS; ***c***) as detailed in Methods. Scale bar: 20 μm.

(22–24, 26). Moreover, because *Xenopus* oocytes express endogenous Nedd4 (27), they are likely to possess all the necessary machinery for proper Nedd4 function.

In our previous work, we demonstrated that rat Nedd4 is expressed in the same cells that express ENaC within the distal nephron and lung epithelia (27, 33), that Nedd4-WW domains bind to the PY motifs of ENaC (27), that point mutations within the PY motifs of ENaC identified in Liddle's syndrome patients (19–21) also abrogate binding to Nedd4-WW domains (27), and finally that ENaC stability and function are regulated by ubiquitination (32). In this study, we present several lines of evidence suggesting that it is indeed Nedd4 that is involved in the ubiquitination and the control of ENaC activity. First, overexpression of xNedd4-wt with ENaC in *Xenopus* oocytes caused a strong inhibition of I_{Na} when compared with oocytes expressing ENaC alone in the presence of endogenous xNedd4. Second, a catalytically inactive form of xNedd4 stimulates these currents, probably by competing with endogenous xNedd4. Third, the effect of xNedd4 is dependent on the presence of intact PY motifs within the ENaC subunits, suggesting an interaction between xNedd4 and the PY motifs, similar to our previous demonstration with rat Nedd4 (27). Moreover, FaNaCh, another channel of the ENaC/degenerin superfamily, which does not contain PY motifs (38), is not regulated by Nedd4.

The current findings provide some interesting insights into the mechanism by which Nedd4 mediates ENaC regulation. The observation that the PY motifs are necessary for Nedd4-dependent regulation strongly suggests that Nedd4 is indeed exerting its effect by binding via its WW domains to the ENaC PY motif. The completely opposite effects on channel function of Nedd4-wt versus the xN4C938S mutant support the hypothesis that Nedd4 is acting via ubiquitination, most likely by ubiquitinating ENaC directly, and thus leading to its retrieval from the plasma membrane and to degradation of the ENaC complex, as is clearly demonstrated by both quantitative surface labeling and immunostaining (Figs. 4 and 5). However, an effect of Nedd4 on ENaC during biosynthesis and recycling processes may also play a role. The inhibition of ENaC internalization in the catalytically impaired xNedd4 mutant is in agreement with our previous findings demonstrating increased channel numbers and increased retention at the cell surface of ENaC bearing Lys→Arg mutations that render the channel ubiquitination defective (32). Recently, Shimkets *et al.* (39) have shown that ENaC is internalized via clathrin-coated pits and that this internalization is dependent on the presence of COOH-terminal endocytosis signals that encompass the PY motifs. It is interesting that a growing number of studies have now demonstrated a link between ubiquitination of transmembrane proteins at the cell surface and their subsequent endocytosis and degradation by the lysosomes or vacuoles in yeast (reviewed in ref. 31). Although it is not yet known how ubiquitination (which normally directs cytosolic or ER proteins to proteasomal degradation) provides a signal for endocytosis of transmembrane proteins, it is quite likely that ENaC also belongs to this class of cell-surface proteins in which ubiquitination and endocytosis and lysosomal degradation are tightly linked.

Our earlier demonstration of binding of Nedd4 to the regions (PY motifs) in ENaC that are deleted/mutated in patients with Liddle's syndrome (27) and our current demonstration of impaired regulation by Nedd4 of ENaC channels lacking one (as in Liddle's syndrome) of its PY motifs clearly implicate Nedd4 in the pathophysiology of this inherited form of hypertension. However, the increased retention of ENaC at the plasma membrane caused by loss of the PY motif, which leads to inhibition of internalization (39) and impaired binding to Nedd4 (thus likely to impaired ubiquitination), cannot provide a full explanation for the defective function of ENaC in Liddle's syndrome. As shown earlier by Firsov *et al.* (25), at least half of the increase in ENaC activity associated with the Liddle's mutations can be attributed to increased open probability of the channel. Whether or not Nedd4 can also affect channel gating is currently unknown. Interestingly, our recent work has demonstrated that ENaC is down-regulated by elevated intracellular Na^+ concentration (26, 40), and that this feedback inhibition is impaired in ENaC chains carrying the Liddle's mutations in their PY motifs (26). Because Nedd4 binds to these PY motifs, it is possible that it is involved in the negative regulation by intracellular Na^+. In support of this notion, a recent report has suggested that Nedd4 mediates the control by intracellular Na^+ of an epithelial Na^+ channel in salivary ducts (41). The molecular identity of the latter channel, however, is unknown; hence deciphering its putative biochemical interactions with Nedd4 is currently not possible.

Despite the strong effect of wild-type or catalytically inactive xNedd4 on ENaC function, a much smaller effect of rat Nedd4 on ENaC activity was observed (data

not shown) even though the protein was expressed at high levels and despite the fact that we used rat ENaC in our experiments. This suggests that either the presence of an additional WW domain in xNedd4 (which contains four WW domains versus three in rNedd4), which may allow better binding to the ENaC tetramer, or the presence of nonconserved NH_2-terminal sequences in xNedd4, which may play a role in localization or functioning of this ubiquitin-protein ligase, could contribute to these differences.

Although thus far there have not been any mutations in Nedd4 identified in patients with Liddle's syndrome, the search for such mutations in inherited forms of hypertension is nevertheless worthwhile in view of our clear demonstration of regulation of the channel by Nedd4. On the other hand, because Nedd4 is likely to have numerous cellular substrates, it is possible that it is an essential gene, as seen in yeast (42), and that mutations that severely interfere with its function may be lethal in higher organisms as well. A gene knockout of Nedd4 in mice, not yet available, may help shed some light on this issue.

In summary, our work provides strong evidence that Nedd4 is a suppressor of ENaC activity that regulates the number of ENaC channels at the plasma membrane. Moreover, it shows that the regulatory effect of Nedd4 requires the interaction between its WW domains and the PY motifs of ENaC, the same motifs that, when mutated, cause Liddle's syndrome. Accordingly, we have demonstrated that this suppressive effect is attenuated in Liddle's syndrome. Thus, this work has implications for our comprehension of the molecular and biochemical mechanisms underlying this disease, as well as for our understanding of ENaC function in general.

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